NOVEL CHIMERIC TNF LIGANDS

TECHNICAL FIELD OF THE INVENTION

[01] The present invention relates to the fields of biochemistry, immunology, genetic engineering, and medicine. In particular, it relates to novel chimeric ligands that, when expressed on the surface of a cell, are more stable than the corresponding native ligand.

BACKGROUND OF THE INVENTION

[02] The immune system eliminates malignant cells by recognizing them as foreign and then clearing them from the body. To accomplish this, the immune system invokes both an antibody response and a cellular response. Both these responses require interaction among a number of different cells of the immune system (Abbas, Cellular and Molecular Immunology, 2000)

[03] An immune reaction typically begins with a T lymphocyte (T cell) that has on its surface a T cell receptor (TCR) that binds to an antigen derived peptide associated with a class II major histo-compatability complex (MHC) molecule. The T cell also expresses on its surface various polypeptides, which are referred to as "ligands" because they bind to receptors on cells associated with an immune-mediated response, as described in more detail below. When the T cell receptor binds to a MHC-associated antigen, such as antigen derived from a malignant cell, it becomes activated and expresses a ligand on its surface. The ligand is only present on the cell surface for a short time, and once it has been removed from the surface of the cell, the T cell's ability to bind a receptor-bearing cell is lost. One such ligand is called tumor necrosis factor (TNFα).

[04] TNFα, when expressed on the surface of an activated T cell, binds to receptors, such as TNF-receptor I (also known as "p55"or "CD120a") and TNF-receptor II (also known as "p75"or "CD120b"), expressed on the surface of immune cells, non-immune cells, and malignant cells. Included among these immune cells are cells collectively referred to as "antigen presenting cells" (APC) because they express surface polypeptides that are able to bind and present antigen to the T cell. Examples of APC include dendritic cells and B cells. APC also have various receptor molecules on their surfaces that interact with other cells of the immune system. The interaction between ligands expressed by T cells and receptor molecules on APC and malignant cells causes a cytolytic reaction that destroys the malignant cells and clears them from the body.

[05] TNFα is one member of a larger family of ligands, collectively referred to as the TNF superfamily (Gruss et al, Cytokines Mol Ther, 1:75-105, 1995 and Locksley et al, Cell, 104:487-501, 2001). Members of the TNF superfamily include Fas ligand ("FasL"), TNFα, LTα, lymphotoxin (TNFβ), CD154, TRAIL, CD70, CD30 ligand, 4-1BB ligand, APRIL, TWEAK, RANK ligand, LIGHT, AITR ligand, ectodysplasin, BLYS, VEGI, and OX40 ligand. TNF superfamily members share a conserved secondary structure comprising four domains: domain I, the intracellular domain; domain II, which spans the cell membrane and is known as the transmembrane domain; domain III, which consists of the extracellular amino acids closest to the cell membrane; and domain IV, the distal extracellular domain (Kipps et al., WO98/26061 published June 18, 1998). Typically, at least a part of domain IV can be cleaved from the parent molecule. The cleaved fragment often exhibits the same biological

activity of the intact ligand and is conventionally referred to as a "soluble form" of the TNF family member.

I. <u>Biological Activity of TNFα</u>

[06] There are two bioactive forms of TNFα. One form is membrane-integrated (mTNFα), also referred to as pro-TNFα. In addition, there is a soluble form (sTNFα) generated by proteolytic cleavage of mTNFα. TNF signals through two distinct receptors, CD120a and CD120b. In general, TNF signaling through CD120a induces cellular apoptosis due to the presence of a cytoplasmic death domain in CD120a. In contrast, CD120b, which lacks a death domain, generally induces cellular activation, such as proliferation and costimulatory molecule expression. These latter effects are highlighted in normal B cells in which TNFα induced expression of important costimulatory molecules, including CD80 and CD54 (Ranheim and Kipps, Cell Immunol. 161:226, 1995).

[07] A matrix metalloproteinase (mmp) called TACE (for TNF-alpha converting enzyme) has been shown to release the soluble form of TNFα (Black et al, Nature, 385:729-733, 1997 and Moss et al, Nature, 385:733-736, 1997). TACE has been found to release sTNFα by cleaving pro-TNFα between amino acid residues alanine76 and valine77. Moreover, this cleavage is dependent on an approximately 12 amino acid mmp recognition sequence spanning valine77 to proline88 (Decoster et al, J Biol Chem, 270:18473-18478, 1995 and Tang et al, Biochemistry, 35:8226-8233, 1996) since deletion of 9 to 12 amino acids of this mmp recognition site inhibited the cleavage of the parent TNFα molecule (Decoster et al, J Biol Chem, 270:18473-18478, 1995 and Perez et al, Cell, 63:251-258, 1990). However, deletion of this cleavage site does not necessarily completely abrogate sTNFα

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generation due to the existence of multiple cleavage sites in TNFα (Mueller et al, J Biol Chem, 274:38112-38118, 1999).

II. Drawbacks of Current TNFα Constructs in Treating Human Diseases

[08] Since TNFα can induce apoptosis of CD120a expressing cells as well as enhance immune responses by cellular activation through CD120b, groups attempted to use TNFα as an anti-tumor compound. However, immune therapy of most cancers with recombinant soluble TNFα showed little clinical efficacy due to the failure to achieve high local concentrations of cytokine without systemic toxicity. Common side effects include fever, chills, anorexia, hypertension, liver abnormalities, and hematological changes (Spriggs et al, Ciba Found Symp, 131:206-227, 1987). Moreover, gene transfer of even wild-type (wt) TNF, expressed as the membrane-associated pro-TNFα, cannot achieve high local expression of TNF without systemic toxicity since it is metabolized rapidly into a soluble cytokine. Since the soluble form of TNFα is the common factor for the failure of TNFα as a therapeutic compound, we hypothesized that design of membrane-stabilized TNFα might allow local delivery of TNFα while mitigating the risk of systemic toxicity associated with soluble TNFα.

[09] Given the disadvantages of current TNFα applications, there is clearly a need for a membrane-stabilized TNFα that maintains the receptor binding function of native TNFα but that is less susceptible to cleavage and is thereby less likely to generate the soluble form of TNFα. The present invention provides such a membrane-stabilized TNFα ligand.

SUMMARY OF THE INVENTION

[010] The present invention relates to novel chimeric TNFα that are more stable when expressed on the surface of cells than non-chimeric TNFα. These novel ligands are chimeric in that they are comprised of domains or subdomains of at least two different members of the TNF superfamily. Specifically, at least one domain or subdomain of TNF that contains a "cleavage site(s)" is replaced with a corresponding domain or subdomain of another ligand of the TNF superfamily, preferably CD154, CD70, FasL or TRAIL. In addition, the chimeric ligand is composed of a domain or subdomain of TNFα that is responsible for binding to the cognate TNFα receptors. The present invention also relates to novel polynucleotide sequences encoding chimeric TNFα, expression vectors comprising the novel polynucleotide sequences, and methods of producing the novel chimeric ligands. Finally, the present invention relates to methods of using the expression vectors to improve the immunoreactivity of transfected cells and to treat malignant tumors.

[011] Thus, one aspect of this invention relates to an isolated polynucleotide sequence encoding a chimeric TNFα, comprising a first nucleotide sequence encoding a domain or subdomain of a tumor necrosis factor ligand other than TNFα, wherein the encoded domain or subdomain replaces a cleavage site of native TNFα, and a second nucleotide sequence encoding a domain or subdomain of native TNFα that binds to a TNFα receptor.

[012] An aspect of this invention is the above isolated polynucleotide sequence wherein the first nucleotide sequence encodes domain III, or a subdomain of domain III, of the other tumor necrosis factor ligand.

[013] An aspect of this invention is the above isolated polynucleotide sequence wherein the first nucleotide sequence additionally encodes domain II, or a subdomain of domain II, of the other tumor necrosis factor ligand.

[014] An aspect of this invention is an isolated polynucleotide sequence such as those described above wherein the first nucleotide sequence additionally encodes domain I, or a subdomain of domain I, of the other tumor necrosis factor ligand.

[015] An aspect of this invention is an isolated polynucleotide sequence such as those described above wherein the first nucleotide sequence additionally encodes a subdomain of domain IV of the other tumor necrosis factor ligand.

[016] An aspect of this invention is an isolated polynucleotide sequence such as those described above, wherein the other tumor necrosis factor ligand is selected from the group consisting of CD154, CD70, Fas ligand and TRAIL.

[017] An aspect of this invention is an isolated polynucleotide sequence such as those described above in which the second nucleotide sequence encodes domain IV, or a subdomain of domain IV, of native TNFα.

[018] An aspect of this invention is an isolated polynucleotide sequence such as those described above in which the second nucleotide sequence encodes a subdomain of domain IV of native TMFα that leaves a cleavage site of native TNFα.

[019] An aspect of this invention is an isolated polynucleotide sequence such as those described above in which the first nucleotide sequence encodes domains I, II and III, or subdomains of one or more of domains I, II and III, of a tumor necrosis factor ligand

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selected from the group consisting of CD154, CD70, Fas ligand, and TRAIL and the second nucleotide sequence encodes domain IV, or a subdomain of domain IV, of native TNFα.

[020] An aspect of this invention is an isolated polynucleotide sequence such as those described above in which the first nucleotide sequence encodes domains I, II and III, or subdomains of one or more domains I, II and III, of CD154 and the second nucleotide sequence encodes domain IV, or a subdomain of domain IV, of native TNFα.

[021] An aspect of this invention is an isolated polynucleotide sequence such as those described above further comprising a linker domain encoding a peptide of at least one amino acid that links the first nucleotide sequence and the second nucleotide sequence.

[022] An aspect of this invention is an isolated polynucleotide sequence such as those described above in which the sequence is selected from the group consisting of SEQ. ID. NO. 1, SEQ. ID. NO. 2, SEQ. ID. NO. 3 and SEQ. ID. NO. 4.

[023] An aspect of this invention is an isolated polynucleotide sequence such as those described above in which the chimeric TNFα comprises an amino acid sequence selected from the group consisting of SEQ. ID. NO. 5, SEQ. ID. NO. 6, SEQ. ID. NO. 7 and SEQ. ID. NO. 8.

[024] An aspect of this invention is a chimeric TNFα comprising a first domain or subdomain of a tumor necrosis factor ligand other than TNFα, wherein the domain or subdomain replaces a cleavage site of native TNFα, and a second domain or subdomain of native TNFα that binds to a TNFα receptor.

[025] An aspect of this invention is a chimeric TNFα that is less susceptible to cleavage from the surface of cells than native TNFα.

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[026] An aspect of this invention is a chimeric TNFα having a cleavage rate that is approximately 90% less than that of native TNFα ligand.

[027] An aspect of this invention is a chimeric TNFα such as those described above in which the domain or subdomain comprises domain III, or a subdomain of domain III, of the other tumor necrosis factor ligand.

[028] An aspect of this invention is a chimeric TNFα such as those described above in which the domain or subdomain further comprises domain II, or a subdomain of domain II, of the other tumor necrosis factor ligand.

[029] An aspect of this invention is a chimeric TNFα such as those described above in which the domain or subdomain further comprises domain I, or a subdomain of domain I, of the other tumor necrosis factor ligand.

[030] An aspect of this invention is a chimeric TNFα such as those described above in which the domain or subdomain further comprises a subdomain of domain IV of the other tumor necrosis factor ligand.

[031] An aspect of this invention is a chimeric TNFα such as those described above in which the other tumor necrosis factor ligand is selected from the group consisting of CD154, CD70, Fas ligand and TRAIL.

[032] An aspect of this invention is a chimeric TNFα such as those described above further comprising domain IV, or a subdomain of domain IV, of native TNFα.

[033] An aspect of this invention is a chimeric TNFα such as those described above comprising a subdomain of domain IV of native TNFα that lacks a cleavage site of native TNFα.

[034] An aspect of this invention is a chimeric TNFα such as those described above comprising domains I, II and III, or subdomains of one or more of domains I, II and III, of a tumor necrosis factor ligand selected from the group consisting of CD154, CD70, Fas ligand and TRAIL, and domain IV, or a subdomain of domain IV, of native TNFα.

[035] An aspect of this invention is a chimeric TNFα such as those described above comprising domain I, domain II and domain III, or subdomains of one or more domains I, II and III, of CD154 and domain IV, of a subdomain of domain IV, of native TNFα.

[036] An aspect of this invention is a chimeric TNFα such as those described above additionally comprising a linker domain that links the first domain or subdomain to the second domain or subdomain.

[037] An aspect of this invention is an expression vector comprising one of the above isolated polynucleotide sequences.

[038] An aspect of this invention is the above expression vector in which the polynucleotide sequence encodes a chimeric TNFα comprising domain III, or a subdomain of domain III, of a tumor necrosis factor ligand selected from the group consisting of CD154, CD70, Fas ligand and TRAIL, and domain IV, or a subdomain of domain IV, of native TNFα.

[039] An aspect of this invention is an expression vector such as those described above further comprising a polynucleotide sequence that encodes domain II, or a subdomain of domain II, of a tumor necrosis factor ligand selected from the group consisting of CD154, CD70, Fas ligand and TRAIL.

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[040] An aspect of this invention is an expression vector such as those described above further comprising a polynucleotide sequence that encodes domain I, or a subdomain of domain I, of a tumor necrosis factor ligand selected from the group consisting of CD154, CD70, Fas ligand and TRAIL.

[041] An aspect of this invention is an expression vector such as those described above further comprising a polynucleotide sequence that encodes a subdomain of domain IV of a tumor necrosis factor ligand selected from the group consisting of CD154, CD70, Fas ligand and TRAIL.

[042] An aspect of this invention is an expression vector such as those described above further comprising a polynucleotide sequence that encodes a subdomain of domain IV of a tumor necrosis factor ligand selected from the group consisting of CD154, CD70, Fas ligand and TRAIL.

[043] An aspect of this invention is an expression vector such as those described above further comprising viral DNA or bacterial DNA.

[044] An aspect of this invention is an expression vector such as those described above further comprising adenoviral DNA, retroviral DNA, or other viral gene transfer system.

[045] An aspect of this invention is an expression vector such as those described above further comprising a promoter region.

[046] An aspect of this invention is an expression vector such as those described above further comprising a polyadenylation signal region.

[047] An aspect of this invention is a genetic construct comprising one of the isolated polynucleotide sequences described above operatively linked to a promoter sequence and to a polyadenylation signal sequence.

[048] An aspect of this invention is a host cell comprising one of the expression vectors or genetic constructs described above.

[049] The above host cell is a mammalian cell in an aspect of this invention.

[050] The host cell is an antigen presenting cell in an aspect of this invention.

[051] The host cell is a tumor cell in an aspect of this invention.

[052] An aspect of this invention is a process for producing a chimeric TNFα comprising culturing one of the above host cells under conditions suitable to effect expression of the protein.

[053] An aspect of this invention is a method for increasing the concentration of a ligand capable of binding to a TNFα receptor on the surface of a cell, comprising introducing into the cell an isolated polynucleotide sequence encoding a chimeric TNFα whereby the chimeric TNFα is less susceptible to cleavage from the surface of the cells than native TNFα.

[054] An aspect of this invention is the above method in which the isolated polynucleotide sequence comprises one of the expression vectors or genetic constructs described above.

[055] An aspect of this invention is the above method in which the cell is a mammalian cell.

[056] An aspect of this invention is the above method in which the cell expresses a TNFα receptor on its surface.

[057] An aspect of this invention is a method for inducing apoptosis in a cell expressing a TNFα receptor comprising introducing into the cell an isolated polynucleotide sequence encoding a chimeric TNFα that is expressed on the surface of the cell.

[058] An aspect of this invention is a method for inducing the activation of an immune system cell comprising introducing into the cell an isolated polynucleotide sequence encoding a chimeric TNFα that is expressed on the surface of the cell.

[059] An aspect of this invention is a method for treating neoplasia in a patient comprising introducing into a neoplastic cell an isolated polynucleotide sequence encoding a chimeric TNFα that is expressed on the surface of the cell.

[060] An aspect of this invention is the above method further comprising obtaining the neoplastic cell from a human patient and infusing the neoplastic cell back into the patient after having introduced into the cells a polynucleotide sequence encoding a chimeric TNFα.

[061] An aspect of this invention is a method of treating neoplasia comprising injecting into a tumor bed of a patient an isolated polynucleotide sequence encoding a chimeric TNFα that is then expressed on the surface of the cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[062] Figure 1. Figure 1 is a schematic diagram of a number of human and mouse ligands of the TNF superfamily depicting domains I-IV of those ligands (Kipps et al., WO98/26061 published June 18, 1998).

[063] Figure 2. Figure 2 is a schematic diagram of wild type TNFα (designated wt TNFα), a deletion mutant of TNFα (designated ^{2}TNFα), and some exemplary TNFα chimeras of the present invention, depicting domains I-IV of those ligands and domain linkers.

[064] Figure 3. Figure 3 is a series of fluorescent activated cell sorting (FACS) histograms showing the comparative surface expression of wt TNFα, the deletion mutant ^{2}TNFα, and some exemplary TNFα chimeras of the present invention on HT1080 cells. The shaded areas represent the background fluorescent staining with isotype-control antibody. The unshaded areas represent the expression level of wt TNFα, the previously described membrane-stabilized ^{2}TNFα, and exemplary chimeric TNFα ligands on the surface of HT1080 cells infected with adenovirus encoding the DNA sequences.

[065] Figure 4. Figure 4 is a series of FACS histograms showing the comparative surface expression of TNFα by uninfected CLL B cells and cells infected with adenovirus encoding wt TNFα and some exemplary TNFα chimeras of the present invention. The shaded areas represent the background fluorescence of isotype-control stained cells. The unshaded areas represent the expression of human TNFα on cells stained with TNF-specific antibody.

[066] Figure 5. Figure 5 shows the quantity of soluble TNFα generated by HT1080 cells infected with wt TNFα adenovirus, ^{2}TNFα adenovirus, and exemplary chimeric TNFα adenovirus vectors, as measured by a TNF-specific ELISA assay.

[067] Figure 6. Figure 6 is a graph representing cell death of WEHI164 cells following infection with adenovirus encoding wt TNFα, ^{2}TNFα, and exemplary chimeric TNFα adenovirus vectors.

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[068] Figure 7. Figure 7 is a diagram showing apoptosis of WEHI164 cells following coincubation with HeLa cells infected with adenovirus encoding CD154:TNFα chimera compared to cells infected with wt TNFα. The darker bar represents apoptosis through cell-to-cell contact, while the lighter bar represents apoptosis mediated by the action of the soluble form of TNFα.

[069] Figure 8. Figure 8 is a series of FACS histograms showing the comparative surface expression of phenotypic markers CD25, CD54, CD96, CD95, and CD70 by CLL B cells following co-culture with HeLa cells expressing wt TNFα and exemplary chimeric TNFα constructs of the present invention.

[070] Figure 9. Figure 9 shows the quantity of soluble TNFα generated by HeLa cells infected with adenovirus vectors encoding wt TNFα, CD154:TNFα chimera, CD154:TNFα containing a putative CD154 mmp recognition sequence at the chimera junction site, or CD154:TNFα lacking the linker domain at the chimera junction site.

[071] Figure 10. Figure 10 shows the quantity of soluble TNFα generated by HeLa cells transfected with plasmids encoding CD70:TNFα chimeras with various modifications made to the linker domain.

[072] Figure 11. Figure 11 shows the percent of tumor bearing mice over time following injection of pre-established tumors with either control adenovirus (LacZ), wt TNFα encoding adenovirus, or CD154:TNFα chimera encoding adenovirus.

DETAILED DESCRIPTION OF THE INVENTION

[073] All cited references are incorporated by reference, including any drawings, as if fully set forth herein.

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I. Definitions

[074] As used herein, the term "chimeric TNFα" refers to a ligand comprised of at least one domain or subdomain of TNFα and at least one domain or subdomain of another TNF ligand other than TNFα.

[075] As used herein, the term "subdomain" refers to a sequence of at least two amino acids that is part of a domain of a TNF ligand. A "subdomain" also encompasses an amino acid sequence from which one or more amino acids have been deleted, including one or more amino acids truncated from an end of the sequence.

[076] As used herein, the term "cleavage site" and "mmp recognition site" refer to a sequence of amino acids that is recognized by proteases, typically matrix metalloproteases (mmp), such as TNFα converting enzyme (TACE), that cleave TNFα from the surface of the expressing cell. TACE has been found to release sTNFα by cleaving pro-TNF between amino acid residues alanine76 and valine77. Moreover, this cleavage is dependent on an approximately 12 amino acid mmp recognition sequence spanning valine77 to proline88. The cleavage site of TNFα is typically found at or around the boundaries of domains III and IV of TNFα.

[077] As used herein, the term "linker domain" refers to a sequence of at least one amino acid that is not part of the native TNFα ligand that joins a domain or subdomain of TNFα chimeric constructs. Although the linker domain is typically two to four amino acids in length as described in our examples, the linker can be any number of amino acids (one amino acid and greater) as long as it does not affect the binding of TNFα chimeric constructs to its cognate receptors. This linker can be composed of noncharged (e.g.

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alanine and glycine) or charged amino acids (e.g. aspartic acid). Moreover, the linker domain is not an absolute requirement in chimeric TNFα constructs since removal of the linker domain should not affect the function or metabolic processing of the TNFα chimeras. The use of linker domains is described in the literature (Ladurner et al, J Mol Biol, 273:330-337., 1997 and Wu et al, Q J Nucl Med, 44:268-283., 2000).

[078] As used herein, the phrase "less susceptible to cleavage" refers to the higher resistance of a chimeric TNFα to proteolytic cleavage compared to that of native TNFα, as measured by the amount of soluble TNF generated by a given number of cells over a period of time. Thus, a chimeric TNFα of the present invention is "less susceptible to cleavage" because it is cleaved at a rate preferably at least 90% less than that of native TNFα.

[079] As used herein, the term "expression vector" refers to a nucleic acid that expresses a recombinant nucleotide sequence and that is capable of infecting cells and replicating itself therein. Typical expression vectors include plasmids used in recombinant DNA technology and various viruses capable of replicating within bacterial or animal cells. A number of expression vectors have been described in the literature. Cantwell et al., Blood, In (1996) entitled "Adenovirus Vector Infection of Chronic Lymphocytic Leukemia B Cells;" Woll, P. J. and I. R. Hart, Ann. Oncol., 6 Suppl 1:73 (1995); Smith, K. T., A. J. Shepherd, J. E. Boyd, and G. M. Lees, Gene Ther., 3:190 (1996); Cooper, M. J., Semin. Oncol., 23:172 (1996); Shaughnessy, E., D. Lu, S. Chatterjee, and K. K. Wong, Semin. Oncol., 23:159 (1996); Glorioso, J. C., N. A. DeLuca, and D. J. Fink, Annu. Rev. Microbiol., 49:675 (1995); Flotte, T. R. and B. J. Carter, Gene Ther., 2:357 (1995); Randrianarison-Jewtoukoff,

V. and M. Perricaudet, Biologicals., 23:145 (1995); Kohn, D. B., Curr. Opin. Pediatr., 7:56 (1995); Vile, R. G. and S. J. Russell, Br. Med. Bull., 51:12 (1995); Russell, S. J., Semin. Cancer Biol., 5:437 (1994); and Ali, M., N. R. Lemoine, and C. J. Ring, Gene Ther., 1:367 (1994).

II. Chimeric DNA Sequences Encoding Chimeric TNFα Ligand

As noted above, ligands of the TNF superfamily ("TNF ligands") have a similar secondary structure consisting of a number of domains (Kipps et al., WO98/76061 published June 18, 1998). In Table I, the domain boundaries of a number of ligands of the TNF superfamily are shown. Based on the x-ray crystal structure of human TNFα, the predicted secondary structure of the receptor-binding portion of CD40 ligand has been deduced (Peitsch et al, Int Immunol, 5:233-238, 1993). The secondary structures of the receptor-binding portions of other TNF ligands were deduced by comparison to human TNFα, using computer analysis.

TABLE I

DOMAIN STRUCTURE OF LIGANDS FROM THE TNF SUPERFAMILY*

	Domain I (Cytoplasmic)	Domain II (Transmembrane)	Domain III (Proximal Extracellular)	Domain IV (Distal Extracellular)
Human CD154	1-42	42-135	135-330	330-786
Murine CD154	1-42	42-135	135-327	327-783

	Domain I (Cytoplasmic)	Domain II (Transmembrane)	Domain III (Proximal Extracellular)	Domain IV (Distal Extracellular)
Bovine CD154	1-42	42-135	135-330	330-786
Human TNFα	1-87	87-168	168-228	228-699
Murine TNFα	1-87	87-168	168-237	237-705
Porcine TNFα	1-87	87-168	168-228	228-696
Human Fas Ligand	1-237	237-315	315-390	390-843
Murine Fas Ligand	1-237	237-309	309-384	384-837
Human CD70	1-45	45-117	117-132	132-579
Human				
CD30 Ligand	1-117	117-186	186-240	240-702
Human TRAIL	1-42	42-111	111-345	345-843

* The domains are identified by the nucleotide boundaries of each domain using the first nucleotide of the initial methionine of the cDNA as nucleotide number 1. According to the invention, the nucleotide boundaries shown may vary considerably from those identified and still define domains that are useful in the present invention.

[080] Given the similarity of structure among TNF superfamily members and the nucleotide sequences coding for them, a nucleotide sequence encoding one domain or subdomain from TNFα should be interchangeable with the corresponding nucleotide sequence of another TNF ligand to result in a hybrid polynucleotide sequence that encodes a chimeric TNFα.

[081] The nucleotide sequences that are exchanged for corresponding sequences in a different TNF ligand gene are selected for functional reasons, i.e., because the new sequence encodes a domain or subdomain that either provides or modifies a desired function, or eliminates an undesired function of the target ligand gene. For example, it is well known that at least part of TNFα is cleaved from the parent molecule and becomes a soluble form. As noted above, the soluble form is generally undesirable. Thus, exchanging a sequence from a TNF ligand that does not contain a cleavage with the cleavage site(s) of TNFα that give rise to the soluble form of TNFα would at least partially ameliorate that problem.

[082] According to the invention, domain III of TNFα includes sequences of amino acids that are cleaved by proteases. For instance, cleavage sites have been identified for TNFα between amino acids ALA76 and VAL77. Cleavage at this site generates a soluble form of the TNFα molecule. As noted above, native TNFα may have additional cleavage sites in domains I-IV(Mueller et al, J Biol Chem, 274:38112-38118, 1999).

[083] Moreover, according to the invention, domain IV of TNFα includes one or more amino acids that are necessary in binding to TNFα receptors and must be conserved to maintain TNFα receptor binding.

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[084] Thus, a presently preferred embodiment of the present invention is a chimeric TNFα polynucleotide sequence comprising a first nucleotide sequence encoding a domain or subdomain of a TNF ligand other than native TNFα, wherein the encoded domain or subdomain replaces the domain or subdomain of native TNFα that contains a cleavage site. Thus, this first sequence may, without limitation, encode any of the following domains, subdomains or combinations thereof: a subdomain of domain III replacing a cleavage site of native TNFα; all of domain III; domain III with domain II or a subdomain thereof replacing a native TNFα cleavage site; domain III with domain I or a subdomain thereof replacing a native TNFα cleavage site; domain III with a subdomain of domain IV replacing a native TNFα cleavage site; domain III, domain II and domain I, or subdomains thereof. Preferably, the first nucleotide sequence encodes at least one domain or subdomain of one of the following TNF ligands: CD154, CD70, FasL and TRAIL. According to the invention, replacing a domain or subdomain containing a TNFα cleavage site with a domain or subdomain from one of these four other TNF ligands results in a chimeric TNFα that is markedly less susceptible to cleavage than native TNFα.

[085] The first nucleotide sequence is operatively linked to a second nucleotide sequence that encodes an extracellular domain or subdomain of native TNFα involved in binding to TNFα receptors. This domain or subdomain comprises all of domain IV of native TNFα or a subdomain thereof that can bind TNF-R1, TNF-R2 or other TNFα receptors. In this way, the chimeric polynucleotide sequence provided by the present invention encodes a chimeric TNFα that binds to cells expressing a TNFα receptor.

[086] A presently preferred polynucleotide sequence encodes a subdomain IV of native TNFα operatively linked to domain I, II and III of another ligand selected from the group consisting of CD154, CD70, FasL and TRAIL. For example, in one presently preferred embodiment, nucleotides encoding a domain IV or subdomain of domain IV of human TNFα is operatively linked to the nucleotides encoding domains I, II and subdomain III of human CD154 that also lacks the CD154 cleavage site (CD154:TNFα). Such a polynucleotide sequence is provided herein as SEQ. ID. NO. 1. Alternatively, the nucleotides encoding subdomain III of human CD154 may include the CD154 cleavage site (designated CD154 + mmp:TNFα). Another example of a presently preferred embodiment is a nucleotide sequence encoding a domain IV or a subdomain of domain IV of human TNFα operatively linked to nucleotide sequences encoding domains I, II, and III of human CD70 (SEQ. ID. NO. 2). SEQ. ID. NO. 3 provides yet another example of a presently preferred polynucleotide sequence, in which a nucleotide sequence encoding domain IV or a subdomain of domain IV of human TNFα is operatively linked to nucleotide sequences encoding domains I, II and III of human FasL. Finally, SEQ. ID. NO. 4, still another presently preferred embodiment of this invention, provides a nucleotide sequence encoding domain IV or a subdomain of domain IV of human TNFα operatively linked to nucleotide sequences encoding domains I, II and III of human TRAIL. In all of these embodiments, the nucleotides preferably encode subdomains of domain IV of human TNFα that lacks a TNFα cleavage site. In addition, domains I, II, and III of the TNF family members described in SEQ. ID. NO's. 1-4 are joined to domain IV of TNFα by a linker domain encoding a peptide from two to four amino acids. The presently most preferred

polynucleotide sequence of the present invention is SEQ. ID. NO. 1. Figure 2 shows domains I-IV of the above-described embodiments of chimeric TNFα. Moreover, the following Table II shows the nucleotide boundaries of these chimeric TNFα sequences.

TABLE II

CONSTRUCT	DOMAINS I-III	DOMAIN IV OF TNF
CD154:TNFα	1-321	265-699
CD70:TNFα	1-132	265-699
FasL:TNFα	1-390	265-699
TRAIL:TNFα	1-345	265-699
CD154 +mmp:TNFα	1-351	265-699

[087] While the above polynucleotide sequences all comprise human TNF ligand sequences, the present invention also contemplates polynucleotide sequences from other species, such as, without limitation, murine polynucleotide sequences.

[088] The encoded chimeric TNFα therefore comprise a polypeptide domain or subdomain of a TNF ligand other than TNFα that replaces a cleavage site of native TNFα. As a result, the chimeric TNFα is less susceptible to cleavage from the surface of cells than native TNFα. Preferably, the exchanged domain is taken from CD154, CD70, FasL or TRAIL. The preferred constructs are: domains I, II and subdomain III of human CD154 and a domain IV or a subdomain of domain IV of human TNFα (SEQ. ID. NO. 5); domains I, II and III of human CD70 and a domain IV or a subdomain of domain IV of human TNFα (SEQ. ID. NO. 6); domains I, II and III of human FasL and a domain IV or a subdomain of

domain IV of human TNFα (SEQ. ID. NO. 7); and domains I, II and III of human TRAIL and a domain IV or a subdomain of domain IV of human TNFα (SEQ. ID. NO. 8). The presently most preferred embodiment for the chimeric TNFα of the present invention is SEQ. ID. NO. 5.

III. <u>Genetic Constructs</u>

[089] The present invention also contemplates an expression vector or any other genetic construct that comprises a polynucleotide sequence of the present invention capable of expressing a chimeric TNFα in a target cell.

[090] An expression vector useful in the present invention contains a polynucleotide sequence encoding a chimeric TNFα operatively linked to a suitable transcriptional or translational regulatory nucleotide sequence, such as one derived from a mammalian, microbial, viral, or insect gene. Such regulatory sequences include sequences having a regulatory role in gene expression, such as a transcriptional promoter or enhancer, an operator sequence to control transcription, a sequence encoding a ribosomal binding site within the messenger RNA, and appropriate sequences which control transcription, translation initiation, or transcription termination.

[091] Particularly useful regulatory sequences include the promoter regions from various mammalian, viral, microbial, and insect genes. The promoter region directs an initiation of transcription through and including the polynucleotide sequence encoding the chimeric TNFα of the present invention. Useful promoter regions include the promoter found in the Rous Sarcoma Virus (RSV) long terminal repeat (LTR), human cytomegalovirus (CMV) enhancer/promoter region, lac promoters, promoters isolated from adenovirus, and any

other promoter known by one of ordinary skill in the art would understand to be useful for gene expression in eukaryotes, prokaryotes, viruses, or microbial cells. Other promoters that are particularly useful for expressing genes and proteins within eukaryotic cells include mammalian cell promoter sequences and enhancer sequences such as those derived from polyoma virus, adenovirus, simian virus 40 (SV40), and the human cytomegalovirus. Particularly useful are the viral early and late promoters, which are typically found adjacent to the viral origin of replication in viruses such as the SV40. One of ordinary skill in the art will understand that the selection of a particular useful promoter depends on the exact cell lines and the other various parameters of the genetic construct to be used to express a polynucleotide sequence within a particular cell line.

[092] Certain genetic constructs contemplated by the present invention therefore include a polynucleotide sequence operatively linked to either a promoter sequence or a promoter and enhancer sequence and also operatively linked to a polyadenylation sequence that directs the termination and polyadenylation of messenger RNA. Preferably, the polynucleotide sequence is constructed using the CMV promoter and the bovine growth hormone polyadenylation sequence.

IV. Host Cells

[093] The present invention also contemplates various host cells that are transformed or transfected with an expression vector or other genetic construct that contains a polynucleotide sequence of the present invention. These cells may be prokaryotic or eukaryotic cells.

[094] In some preferred embodiments the cells are normal antigen presenting cells of a mammal, such as monocytes, macrophages, B cells, and the like. In other preferred embodiments, the cells may be normal cells that are capable of stimulating bystander antigen presenting cells when a polynucleotide sequence of the present invention is introduced into these cells. The present invention also contemplates somatic cells that are not naturally capable of presenting antigen to the immune system but may be genetically engineered with the genes encoding the molecules required for antigen presentation, and thus allow these cells to act as artificial antigen presenting cells. A polynucleotide sequence encoding a chimeric TNFα may then be introduced into these artificial antigen presenting cells. Various tests are well known in the literature to determine whether a particular cell is able to function as an antigen presenting cell, such as cell proliferation or the production of lymphokines, and therefore this aspect of the present invention may be easily determined.

[095] In addition to the above normal human cells, the present invention also contemplates introducing a polynucleotide sequence encoding a chimeric TNFα into various neoplastic or malignant cells, such as cells of the immune system and solid tumors. Such neoplastic cells that are contemplated include leukemia cells, such as acute monocytic leukemia (AML), acute myelomonocytic leukemia (AMML), chronic lymphocytic leukemia (CLL), chronic myelogenous or chronic myelomonocytic leukemia (CMML). Also contemplated are cells derived from lymphomas, gliomas, breast, cervical, ovarian, lung, bladder, or prostate cancers.

[096] Finally, in a preferred embodiment of the present invention, a polynucleotide sequence encoding a chimeric TNFα is introduced into cells that express the cognate receptors for TNFα, such as TNF-R1 and TNF-R2, on surfaces of the cells.

V. Methods Utilizing Genetic Vectors and Constructs Containing an Accessory Molecule Ligand Gene

[097] Recognizing the interaction of TNFα and its cognate receptors in regulating the immune response, the present invention also contemplates methods of increasing the concentration of a membrane-stabilized ligand capable of binding to TNF-R1, TNF-R2, or some other cognate receptor for TNFα, by introducing a polynucleotide sequence encoding a chimeric TNFα into a cell, whereby the chimeric TNFα is less susceptible to cleavage from the surface of that cell relative to native TNFα. Because the chimeric TNFα is less susceptible to proteolytic cleavage, it has increased capacity to bind to its cognate receptor and induce either a cytolytic response or an immune response. In addition, the capacity of cells transfected with a polynucleotide sequence encoding a chimeric TNFα ligand of the present invention to induce apoptosis and clearance of cells bearing a TNFα receptor is increased.

[098] The present invention is useful for any human cell that participates in an immune reaction either as a target for the immune system or as part of the immune system's response to the foreign target. The methods include ex vivo methods, in vivo methods, and various other methods that involve injection of polynucleotides or vectors into the host cell. The methods also include injection directly into the tumor or tumor bed.

[099] The present invention thus contemplates ex vivo methods comprising isolation of cells from an animal or human subject. A polynucleotide sequence encoding a chimeric TNFα of the present invention is introduced into the isolated cells. The cells are then re-introduced at a specific site or directly into the circulation of the subject. In a preferred embodiment of the present invention, cell surface markers, including molecules such as tumor markers or antigens that identify the cells, may be used to specifically isolate these cells from the subject.

[0100] The present invention also contemplates introducing a polynucleotide sequence encoding a chimeric TNFα into the desired cells within the body of an animal or human subject without first removing those cells from the subject. Methods for introducing polynucleotide sequences into specific cells in vivo, or within the subject's body are well known and include use of expression vectors and direct injection of various genetic constructs into the subject. In a typical application, an expression vector containing a polynucleotide sequence of the present invention is introduced into the circulation or at a localized site of the subject to allow the vector to specifically infect the desired cells. In other preferred embodiments the vector is injected directly into the tumor bed present in a subject that contains at least some of the cells into which the polynucleotide sequence of the present invention is to be introduced.

[0101] The present invention also contemplates directly injecting into an animal or human subject a genetic construct that includes a polynucleotide sequence encoding a chimeric TNFα, and may additionally include a promoter and a polyadenylation sequence. Examples of such useful methods have been described (Vile et al, Ann Oncol, 5:59-65,

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1994). The genetic construct may also be directly injected into the muscle or other sites of an animal or human subject or directly into the tumor or tumor bed of the subject.

VI. Methods of Treating Neoplasia

[0102] The present invention is also directed to gene transfer of a polynucleotide sequence encoding a chimeric TNFα of the present invention to induce apoptosis of tumor cells. In addition to directly causing apoptosis of these tumors through interactions between TNFα and its receptors TNF-R1 and TNF-R2, the present invention also contemplates infecting tumor cells with a chimeric TNFα so that the ligand is expressed in a membrane-stabilized manner and thereby may also participate in the immune response.

[0103] Thus, the present invention contemplates methods of treating neoplasia, comprising inserting into a neoplastic cell a polynucleotide sequence of the present invention, so that the encoded chimeric TNFα is expressed on the surface of the neoplastic cells. The present invention contemplates treating human neoplasia both in vivo and ex vivo.

[0104] In a preferred method of treating neoplasia, the method further comprises the steps of first obtaining the neoplastic cells from a subject, inserting therein a polynucleotide sequence of the present invention so that a chimeric TNFα is expressed on the surface of the neoplastic cells, and re-administering the cells back into the subject. One of ordinary skill in the art will understand that numerous methods are applicable for re-administering the transformed neoplastic cells into the subject.

a. EXAMPLES

I. Construction of a Genetic Construct and Gene Therapy Vector Containing a Chimeric Accessory Molecule Ligand Gene

[0105] The chimeric accessory molecule ligand genes of SEQ ID NO. 1 - SEQ ID NO. 4 were constructed and cloned as follows:

i. Preparation of Chimeric Accessory Molecule Ligand Gene Utilizing Domains From Two Different Accessory Molecule Ligand Genes

[0106] DNA fragments encoding domains I-III of a ligand (CD154, CD70, FasL, and TRAIL) were amplified from the full-length cDNA template by PCR using oligonucleotide primers specific for 5′ and 3′ regions flanking domain I-III of the ligand. In addition, a DNA fragment encoding subdomain IV of TNFα was PCR amplified. A BamHI restriction endonuclease site was engineered into the domain III-IV junction PCR primer set to enable ligation of the domain I-III fragment with domain IV fragment. In addition, restriction endonuclease sites were added to the 5′ and 3′ primers that flank domains I and IV, respectively, allowing for ligation into the pcDNA3 vector. Following PCR amplification of the domain I-III fragment and the domain IV fragment, the DNA fragments were digested with BamHI and restriction enzyme corresponding to the 5′ or 3′ flanking regions of domains I and IV. Following digestion, the domain I-III fragment and domain IV fragment were ligated at the BamHI site in parallel to ligation into the multiple cloning site of the eukaryotic expression plasmid pcDNA3 (Invitrogen, San Diego, CA). The chimeric TNFα (hereinafter in the examples, TNFα will be referred to simply as "TNF") DNA insert was

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flanked by the strong-heterologous CMV promoter and the bovine growth hormone polyadenylation sequence.

ii. Adenovirus Synthesis

[0107] The chimeric TNF-pcDNA3 plasmids were digested with the restriction enzymes *NruI* and *Sma I* to release a DNA fragment containing the CMV promoter from pCDNA3, the chimeric TNF gene, and the polyadenylation signal from pCDNA3. Following gel purification of this fragment by separation of the digested DNA on a 1% agarose gel, the DNA fragment was ligated into the *EcoRV* site of the adenoviral shuttle vector MCS (SK) pXCX2. This plasmid is a modification of the plasmid pXCX2 such that the pBluescript polylinker sequence has been cloned into the E1 region, (J. R. Tozer, UCSD, unpublished data, September 1993). Following purification of chimeric TNF-MCS (SK) pXCX2 plasmid, 5ug of this shuttle plasmid was cotransfected with 5ug of JM17 plasmid into 293AC2 cells using the calcium phosphate Profection Kit from Promega according to the manufacturer's instructions. Following transfection, the cells were cultured for 5 days to allow for homologous recombination and viral synthesis. Total cells and supernatant were then harvested and freeze-thawed thrice to release cell-associated adenovirus.

[0108] Following the initial viral production, a clonal isolate of the virus obtained by plaque purification. Briefly, the freeze-thawed viral supernatant was cleared of debris by centrifugation at 1000 rpm in a tabletop centrifuge for 5 minutes. 293AC2 cells grown to confluency in 6 well tissue culture plates were then infected with serial dilutions of the viral supernatant for 1-2 hours. Following infection, the media was aspirated and cells overlayed with DMEM media containing 4% fetal calf serum and 0.65% agarose held at

56°C. Following 4-6 days incubation, isolated plaques were picked into 1ml of media and subsequently used for viral amplification.

[0109] Large-scale adenovirus preparations were prepared by successively infecting increasing quantities of 293AC2. Purified adenovirus was then purified over cesium chloride step gradients. This method makes use of a cesium chloride gradient for concentrating virus particles via a step gradient, with the densities of 1.45g/cm^3 and 1.20g/cm^3, in which 293AC2 expanded virus samples are centrifuged for 2 hours in a SW40 rotor (Beckman, Brea, CA) at 25,000 rpm at 4° C. The virus band was isolated using a 27-gauge needle and syringe and desalted using a Sephadex G-25 DNA grade column (Pharmacia, Piscataway, NJ). The virus was desalted against phosphate-buffered saline containing 10% glycerol and stored at –70°C. The final titer of the virus was determined by anion-exchange HPLC.

II. Introduction and Expression of a Chimeric Accessory Molecule Ligand Gene in CLL Cells and HeLa Cells

i. Expression

[0110] TNFα surface expression was detected by flow cytometry. Briefly, the adherent cells were detached from the wells aspiration of the media and addition of detaching solution (PBS containing 10mM EDTA, pH 8). Once the cells detached from the plate, half of each sample was analyzed for ligand expression by flow cytometry. Briefly, cells were washed once in FACS staining buffer (composed of PBS containing 3% FCS and 0.05% sodium azide), resuspended in FACS buffer to approximately 10^7 cells/ml, and $5x10^5$ (50ul) cells were plated in 96-well u-bottom plastic microwell plates. For human TNFα

specific staining, PE-conjugated antibody specific for TNFα (Pharmingen) was added for 30 minutes at 4°C. The cells were then washed twice with FACS buffer, resuspended in FACS buffer, and transferred to FACS tubes for data acquisition. To control for nonspecific antibody binding, all samples were stained with appropriate isotype control antibodies. Furthermore, dead cells and debris were excluded from analysis by addition of 10ng/ml propidium iodide to all staining reactions. The cells were analyzed by flow cytometry for TNFα expression using a FACSCaliber flow cytometer (Becton Dickinson).

[0111] (Figure 3) shows the expression of different chimeric TNF constructs compared to wild type TNF and a previously described membrane-stabilized TNF (designated □TNF) following adenovirus infection of HT1080 cells. Briefly, HT1080 cells were infected with increasing titers of adenovirus, indicated above histogram columns. Two days following infection, cells were analyzed for TNF surface expression by flow cytometry. This data shows the adenovirus vectors encoding the chimeric TNF constructs were expressed on the cell surface as detected using a fluorochrome-conjugated antibody specific for TNF. In addition, this data shows there were differences in the surface expression levels between TNF constructs. Specifically, Ad-CD154:TNF infection resulted in the highest levels of surface expression of TNF. Similar patterns of expression were obtained in a panel of other cell lines, including 293, HeLa, COLO205, A549, HCT15, PC3, RPMI8226, and BT20 suggesting the differences in expression between the TNF constructs are not cell type restricted.

[0112] (Figure 4) shows the expression of different chimeric TNF constructs following adenovirus infection of chronic lymphocytic leukemia (CLL) B cells. CLL cells were

infected with adenovirus, as indicated above each histogram, at a multiplicity of infection (M.O.I.) ratio of 1000. Two days following infection, $CD19^+$ B cells were examined for TNF surface expression by flow cytometry. In addition, the figure shows the same pattern of expression differences between TNF constructs that we observed for cell lines described above. Namely, TNF chimera expression was greater than wild type TNF. Again, the greatest TNF surface expression was obtained with the hCD154:TNF chimera.

ii. <u>Generation of Soluble Molecules</u>

2. Soluble TNF Generation: ELISA Quantitation

(Figure 5) shows the quantity of soluble TNF generated by HT1080 cells infected with chimeric TNFα adenovirus vectors. Cells were infected at a M.O.I. ratio of 10. Two days following infection, supernatant was harvested and cleared of dead cells and debris by centrifugation. Soluble TNF was measured by enzyme linked immunosorbent assay (ELISA) using a TNF-specific ELISA assay from Pharmingen, Inc. (La Jolla, CA) according to the manufacturer's instructions. Specific quantities of TNF were calculated based on titrations of a known quantity of recombinant TNF (Biosource International). This data shows the chimeric TNF constructs generated significantly less soluble TNF than either wild type TNF (wt TNF), or the previously described membrane-stabilized ▯TNF lacking the putative mmp proteolytic site. Moreover, despite the highest surface expression levels of CD154:TNF compared to all other constructs, CD154:TNF generates the least soluble TNF. This pattern of soluble TNF release was also observed for other cell lines, including HeLa, 293, A549, COLO205, HCT-15, and BT-20.

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iii. Functional Assays of Chimeric Accessory Molecule Ligands

1. TNF Chimera Killing of WEHI164 Fibrosarcoma Cells: Coculture Assay

[0113] (Figure 6) demonstrates TNF chimeras are functional using a biological apoptosis assay previously described (Espevik et al, J Immunol Methods, 95:99-105, 1986). Following infection of HeLa cells with adenovirus for two days at a M.O.I. ratio of 10, WEHI164 cells, a TNF sensitive cell line, were overlayed on the infected HeLa cells and incubated an additional 18hr. The WEHI164 cells were prelabelled with PKH26 (Sigma, Inc.), a red fluorescent chemical that enables gating the WEHI164 cells from the HeLa cells. WEHI cells were stained with propidium iodide and analyzed for cell death by flow cytometry. This data shows that WEHI cells were killed following coculture with TNF-expressing HeLa cells.

2. Cell Contact Dependent Apoptosis of WEHI164 By TNF Chimera

[0114] (Figure 7) demonstrates contact dependent killing of WEHI164 cells by TNF chimera. This demonstrates membrane-stabile expression of the TNF chimera. Briefly, HeLa cells were infected with adenovirus for one day at a M.O.I. ratio of 10. WEHI164 cells were then mixed directly with the infected HeLa cells or separated from the HeLa cells by a 0.2 micron transwell insert. This insert prevents direct cell-cell contact but permits diffusion of soluble molecules (e.g. soluble TNF) between cells. 18hr following mixing, the WEHI164 cells were analyzed for apoptosis as described in figure 6. In contrast to wt TNF that released soluble TNF that could kill WEHI164 cells separated by

the transwell insert, the TNF chimera did not release soluble TNF that could similarly induce apoptosis.

3. TNF Chimera Activation of CLL B Cells

[0115] (Figure 8) shows the activation of CLL B cells cocultured with HeLa cells expressing chimeric TNF. HeLa cells were infected with adenovirus at a M.O.I ratio of 10. Two days following infection, CLL cells were overlayed on the HeLa cells and co-incubated for 1 day. $CD19^+$ CLL cells were then analyzed for changes in expression of phenotypic markers (CD25, CD54, CD86, CD95, and CD70). Bold-line histograms represent CLL cells cocultured with Ad-TNF vector, as labeled to the left of each row. Thin-line histograms represent coculture with Ad-LacZ virus. Shaded histograms represent staining with an isotype control monoclonal antibody of irrelevant specificity. This data shows that chimeric TNF constructs are functional in that they modulated expression of a panel of phenotypic markers on CLL cells characteristic of lymphocyte activation.

4. Modified mmp Site TNF Chimera Soluble TNF Generation

[0116] (Figure 9) shows the quantity of soluble TNF generated by HeLa cells infected with chimeric CD154:TNFα adenovirus vector containing the putative CD154 mmp recognition site that is absent from the CD154:TNF chimera described in Figures 3-8. This construct is termed CD154 +mmp:TNF (SEQUENCE ID#9). Cells were infected with adenovirus at a M.O.I. ratio of 10. Two days following infection, supernatant was harvested and cleared of dead cells and debris by centrifugation. Soluble TNF was measured by enzyme linked immunosorbent assay (ELISA) using a TNF-specific ELISA assay from Pharmingen, Inc. (La Jolla, CA) according to the manufacturer's instructions. Specific quantities of TNF were

calculated based on titrations of a known quantity of recombinant TNF (Biosource International). This data shows the modifications described above to the original CD154:TNF chimera did not affect their susceptibility to proteolytic cleavage into a soluble molecule.

5. Modified Linker Domain Effect on Soluble TNF Generation

[0117] (Figure 10) shows the quantity of soluble TNF generated by HeLa cells transfected with plasmids encoding CD70:TNF chimeras with various modifications made to the linker domain. In addition to the CD70:TNF construct described in figures 3-4, constructs with a truncated linker domain (□Linker CD70:TNF, Sequence ID#10) and with a linker domain containing a modified amino acid sequence (Linker$^{DP\text{->}GA}$ CD70:TNF, Sequence ID#11) are shown. HeLa cells were transfected with plasmid using Lipofectamine2000 (Gibco-BRL) according to the manufacturer's instructions. Two days following transfection, supernatant was harvested and soluble TNF was measured by ELISA as described above. This data shows that modifications to the linker domain of TNF chimeras do not affect the stability of these constructs.

6. Treatment of Pre-established Murine WEHI164 Tumors With Chimeric TNF

[0118] (Figure 11) shows the percent of tumor bearing mice with pre-established WEHI164 tumors following intratumoral injection with adenovirus encoding either β-galactosidase (LacZ), wt TNF, or chimeric CD154:TNF. Briefly, Balb/c mice were inoculated subcutaneously with $3x10^6$ WEHI164 cells and tumor nodules were allowed to form for 10 days. On days 10, 12, and 14 following tumor inoculation, $5x10^8$ plaque forming units (pfu) of virus was delivered by intra-tumoral injection. Animals were then monitored

weekly for tumor presence. Animals were euthanized when the tumor diameter reached >2cm. This data shows that 75% of mice treated with chimeric CD154:TNF had complete tumor regression, compared to tumor regression in only 50% of mice treated with wt TNF. There was no tumor regression in mice treated with control adenovirus (Ad-LacZ). This data suggests chimeric TNF is therapeutically active against tumors and this activity is greater than wt TNF.

[0119] While preferred embodiments have been shown and described, it will be apparent to one of ordinary skill in the art that numerous alterations may be made without departing from the spirit or scope of the invention. The invention is not to be limited except in accordance with the following claims and their legal equivalents.